



# Detection of *MPLW515L/K* Mutations and Determination of Allele Frequencies with a Single-Tube PCR Assay

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## Abstract

A gain-of-function mutation in the *myeloproliferative leukemia virus (MPL)* gene, which encodes the thrombopoietin receptor, has been identified in patients with essential thrombocythemia and primary myelofibrosis, subgroups of classic myeloproliferative neoplasms (MPNs). The presence of *MPL* gene mutations is a critical diagnostic criterion for these diseases. Here, we developed a rapid, simple, and cost-effective method of detecting two major *MPL* mutations, *MPLW515L/K*, in a single PCR assay; we termed this method DARMS (dual amplification refractory mutation system)-PCR. DARMS-PCR is designed to produce three different PCR products corresponding to *MPLW515L*, *MPLW515K*, and all *MPL* alleles. The amplicons are later detected and quantified using a capillary sequencer to determine the relative frequencies of the mutant and wild-type alleles. Applying DARMS-PCR to human specimens, we successfully identified *MPL* mutations in MPN patients, with the exception of patients bearing mutant allele frequencies below the detection limit (5%) of this method. The *MPL* mutant allele frequencies determined using DARMS-PCR correlated strongly with the values determined using deep sequencing. Thus, we demonstrated the potential of DARMS-PCR to detect *MPL* mutations and determine the allele frequencies in a timely and cost-effective manner.

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## Introduction

Detecting clonal mutations and measuring mutant allele frequencies in malignant tissues are important tasks in clinical laboratories. In patients with essential thrombocytosis (ET) and primary myelofibrosis (PMF), acquired mutations in genes such as *JAK2* tyrosine kinase, *calreticulin (CALR)*, and *myeloproliferative leukemia virus (MPL)* have been described [1–3]. *JAK2* and *MPL* mutations have been demonstrated to play causal roles in MPN development in animal models [4,5]. Based on these findings, the identification of clonal mutations was deemed a diagnostic criterion for MPN by the World Health Organization (WHO) in 2008.

The major *JAK2* mutation found in MPN patients is *JAK2V617F* (G1849T); therefore, epidemiological studies of MPN examining *JAK2V617F* have been greatly facilitated by PCR-based assays, such as ARMS (amplification refractory mutation system)-PCR [6], ABC (alternately binding probe competitive)-PCR [7], and allele specific (AS)-qPCR [8–11]. In contrast, a gain-of-function mutation in *MPL*, which encodes the thrombopoietin (TPO) receptor, has been identified in multiple

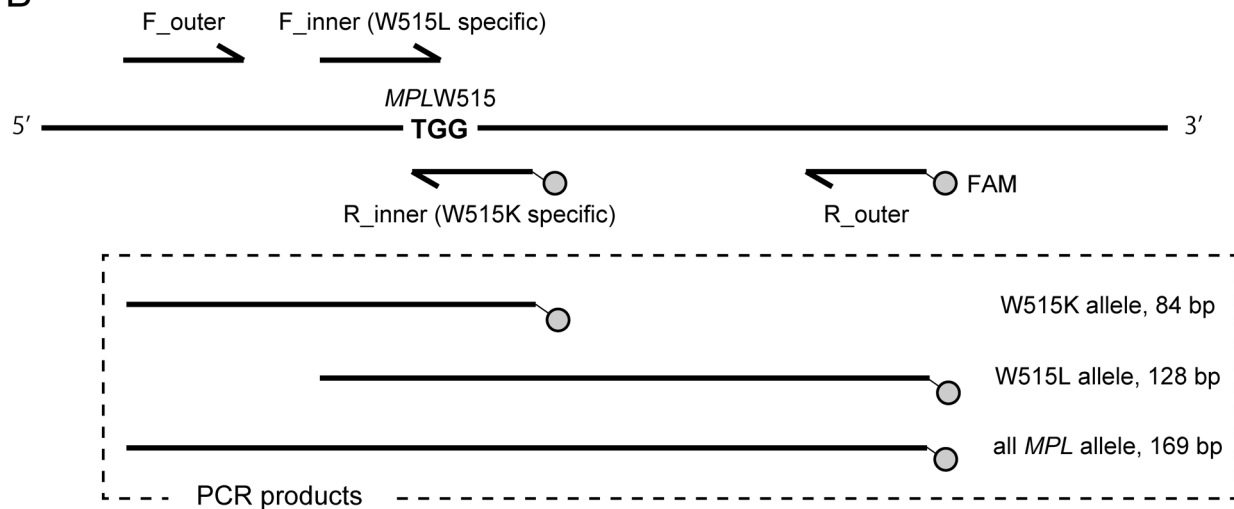
sites within exon 10, making its detection with a simple PCR-based assay difficult. *MPL* mutations in MPN patients have traditionally been characterized using melting curve assays combined with Sanger sequencing [12,13], quantitative-PCR [14,15], or, more recently, deep sequencing [16,17]. However, these assays generally require expensive machines and reagents and greater workloads. Partly because of the absence of a practical method for detecting *MPL* mutations, there have been many more epidemiological studies of MPN patients carrying the *JAK2* V617F mutation compared with *MPL* mutations.

Thus far, 10 types of *MPL* mutations have been identified in or around the transmembrane domain of *MPL* [17–19], and most of these result in the activation of *MPL*, even in the absence of TPO, and the subsequent activation of downstream targets [5,20,21]. These mutations include G1544T and TG1543\_1544AA, which result in the substitution of tryptophan with leucine (W515L) or lysine (W515K), respectively, and are found in nearly three-quarters of patients bearing *MPL* mutations [17]. *MPLW515L/K* has been shown to promote tumorigenesis *in vivo* [5], and a higher mutant allele frequency is associated with progression to myelofibrosis [17]. Thus, detecting *MPLW515L/K* mutations and

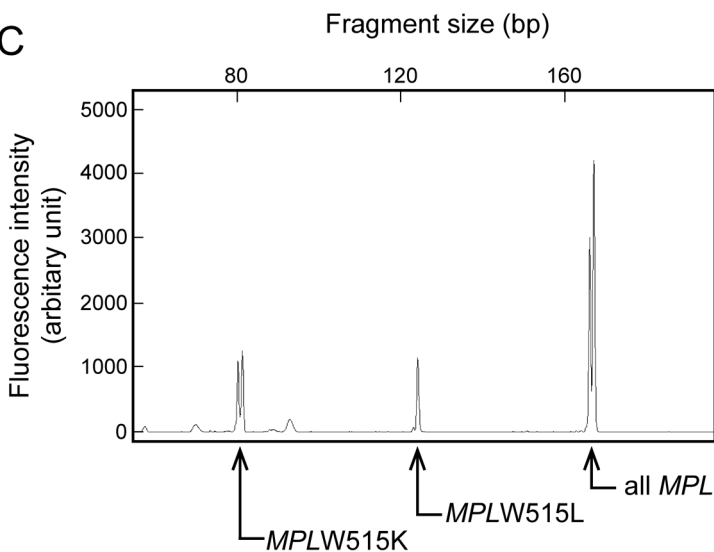
A

Name	Description	Sequence (5' → 3')
F_outer	Common forward primer	agtaggggctggctggat
F_inner	W515L specific forward primer	ggcctgctgctgctgaAgtt
R_inner	W515K specific reverse primer	FAM - acctgtagtgtcaggaaactTc <u>tt</u>
R_outer	Common reverse primer	FAM - ggtcacagagcgaaccaaga

B



C



**Figure 1. Detection of *MPLW515L/K* mutations using DARMS-PCR.** (A) The primers used in the DARMS-PCR assay. The two inner primers harbored sequences (underlined) that matched *MPLW515L* or *W515K*, but not the wild-type allele. Other mismatches (capital letters) were introduced into the inner primers to reduce the annealing of the mutant-specific primers to the wild-type sequence. The reverse primers were labeled with FAM (5-carboxyfluorescein hydrate) at the 5' terminus. (B) A schematic representation of DARMS-PCR products. The two outer primers were designed to generate a 169-base-pair (bp) PCR product from all *MPL* alleles. The F\_inner and R\_inner primers annealed specifically to the *MPLW515L* and *W515K* alleles, respectively; in combination with the outer primers, they generated 84- and 128-bp PCR products, respectively. From a mutant allele, both 169-bp and 84- or 128-bp fragments were amplified, while, only the 169-bp fragment was generated from the wild-type allele. (C) Demonstration of DARMS-PCR. A capillary electropherogram of DARMS-PCR products showing three peaks derived from wild-type *MPL*, *W515L*, and *W515K*. This result was obtained when PCR was performed with a standard DNA mixture containing equal ratios of *MPL* wild-type, *W515L*, and *W515K* alleles with a total copy number of  $10^5$ . The horizontal axis represents the fragment length, and the vertical axis represents the fluorescence intensity.  
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determining the relative ratios of W515L or W515K to the wild-type allele is critical for improving our understanding of MPN pathogenesis. In this work, we developed a cost-effective and practical method for simultaneously detecting both the *MPLW515L* and *W515K* mutations with high sensitivity and for determining the *MPLW515L/K* allele frequencies using a single PCR assay.

## Materials and Methods

### Standard DNA preparation

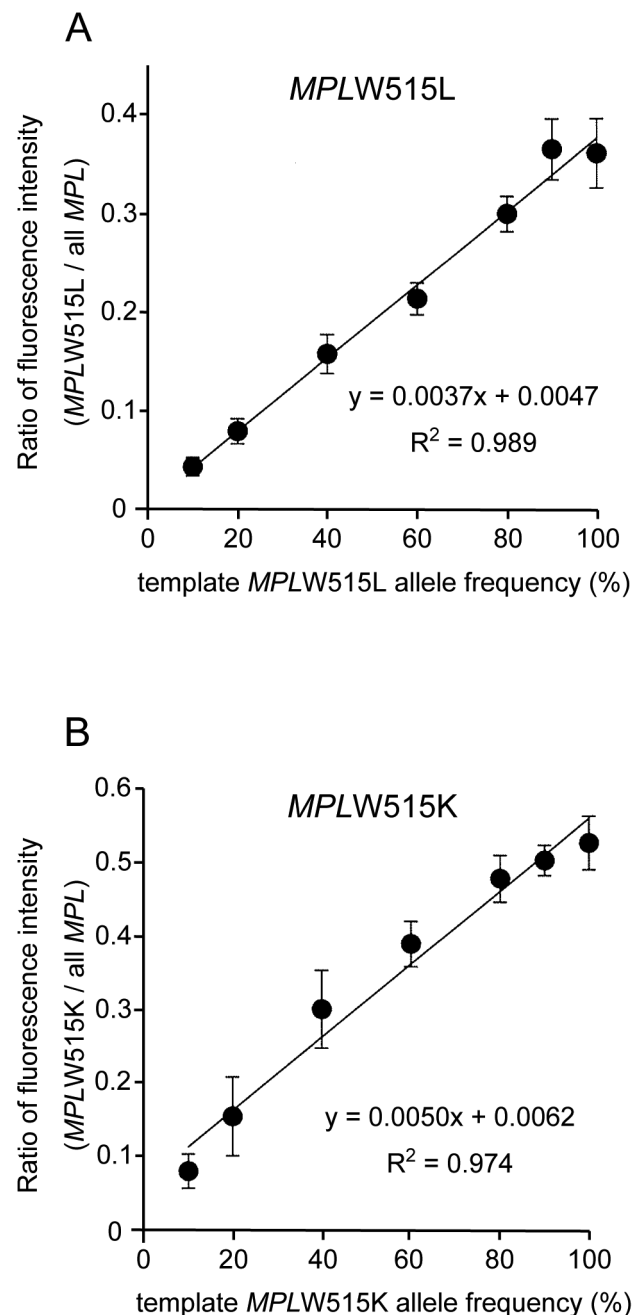
A 1,331-base-pair (bp) fragment containing the human *MPL* sequence (43348414 to 43349744 of NC\_000001.11) was PCR-amplified from human genomic DNA (#G3048 Promega, Dane County, USA) with a set of primers (forward primer: AAATCTGGCATCCTCTGCAGCATGAGTATTATTTG; reverse primer: CAAGAGGTTCTGTTTCAGTGAGTCAGGTCGTGT). The PCR product was subcloned into the pSP73 cloning vector (Promega) to generate pSP73/*MPL*-WT. An *MPLW515L* (G1544T) or *W515K* (TG1543\_1544AA) mutation was introduced into pSP73/*MPL*-WT using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer's instructions. The sequences of all of the plasmids were confirmed via Sanger sequencing. A 3,339-bp plasmid carrying the wild-type *MPL*, *W515L*, or *W515K* DNA sequence was linearized by *ScaI* restriction enzyme digestion, subjected to agarose gel electrophoresis, and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified DNA was mixed at the indicated ratio (see Results and Discussion) to create a standard template for dual amplification refractory mutation system (DARMS)-PCR.

### DARMS-PCR

*MPL* sequences, including TGG1543\_1545 (encoding wild-type *W515*), were amplified via ARMS-PCR [6] with the following modifications. The reaction mixture (20  $\mu$ L) was composed of 1X AmpliTaq Gold Master Mix (Life Technologies), 120 nM of two outer primers (F\_outer and R\_outer), 1  $\mu$ M of F\_inner primer, 300 nM of R\_inner primer, and template DNA (genomic DNA ranging from 19 to 253 ng). Note that with DARMS-PCR, the frequency of a given *MPL* mutant allele is determined by comparing the relative signal peak values of that mutant and all *MPL* alleles; thus, in theory, the template copy number can be variable. Indeed, we confirmed that using the abovementioned range of standard DNA template, the resulting mutant frequencies did not differ significantly from each other (data not shown). The primer sequences and positions are presented in Figure 1A and B. The PCR conditions were as follows: an initial denaturation at 95°C for 10 min; 28 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec; and a final extension at 72°C for 7 min.

### Detection of *MPL* mutant alleles and determination of allele frequencies

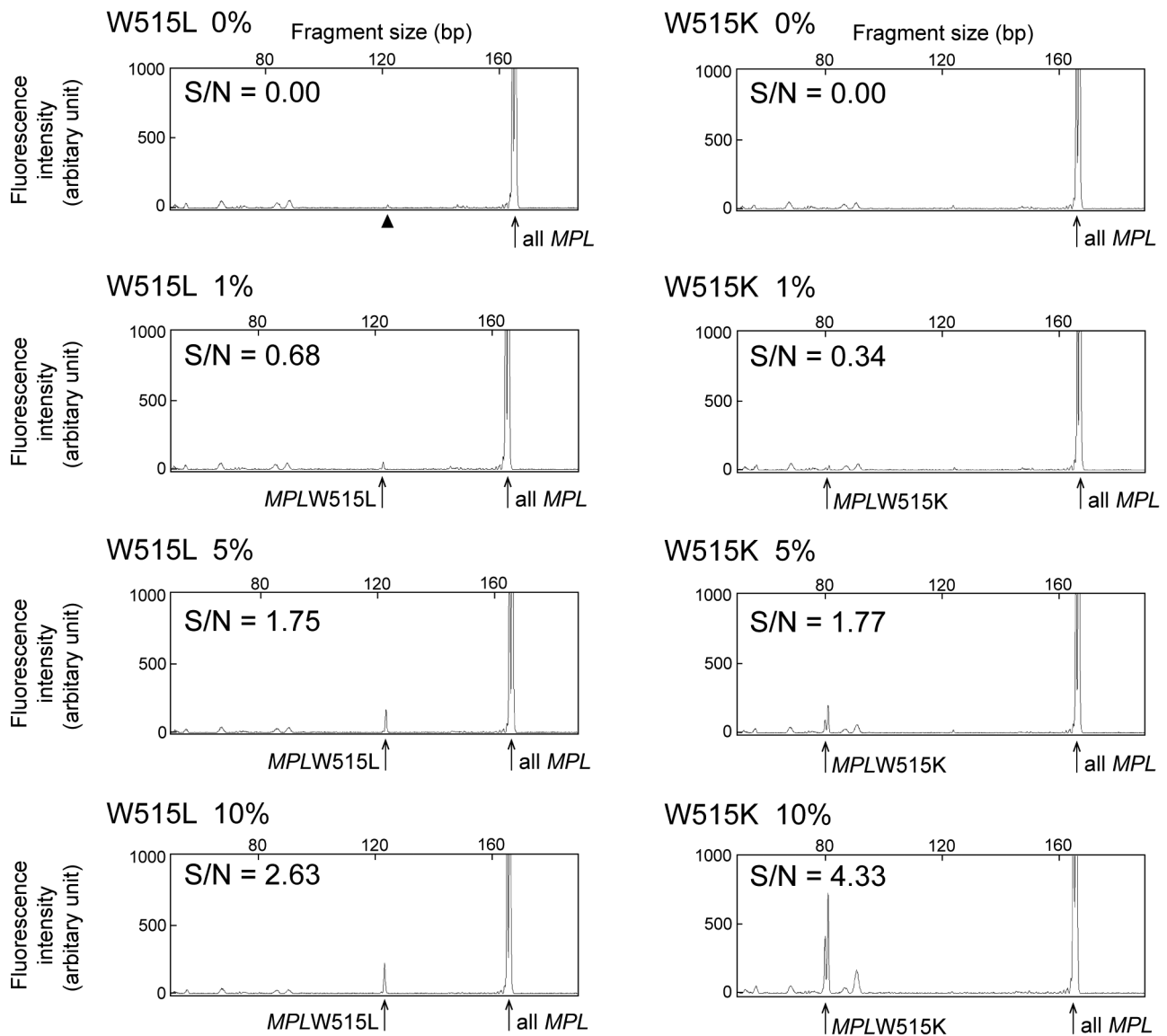
DARMS-PCR products were analyzed via capillary electrophoresis. A capillary electrophoresis mixture was created that contained 1.0  $\mu$ L of six-fold diluted DARMS-PCR products, 0.2  $\mu$ L of GeneScan 500 LIZ Size Standard (Life Technologies, Carlsbad, USA), and 15.8  $\mu$ L of Hi-Di formaldehyde (Life Technologies). A 17- $\mu$ L aliquot of the mixture was heated at



**Figure 2. Demonstration of DARMS-PCR to determine *MPL* mutant allele frequencies.** Standard curves for the quantification of *MPLW515L* (A) and *W515K* (B) allele frequencies. A series of templates containing different amounts of mutant allele DNA were used for DARMS-PCR. The fluorescence peak value of the mutant allele over the value for all *MPL* alleles was calculated for each template. The mean values and errors obtained from three independent experiments are shown.

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86°C for 3 min, cooled on ice, and loaded onto an ABI 3130xl Genetic Analyzer (Life Technologies). The sizes of the PCR products were determined based on the size standard. The height



**Figure 3. Lower limit of *MPL* mutation detection by DARMS-PCR.** Capillary electropherograms for DARMS-PCR assays with low levels of *MPLW515L* or *W515K* mutant alleles are presented. The average fluorescence intensities for nonspecific PCR products were defined as noise. S/N ratios, which were calculated as the fluorescence peak value for a mutant PCR product over the value for noise, are shown. An arrow head in the *W515L* 0% panel indicates a false PCR product (see Results and Discussion).  
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of each fluorescence peak corresponding to wild-type *MPL*, *W515K*, or *W515L* was measured. The fluorescence peak values of a standard DNA mixture containing a known wild-type-to-mutant *MPL* ratio were used to generate the following formulas for calculating the *MPL* mutant allele frequencies (see Results and Discussion).

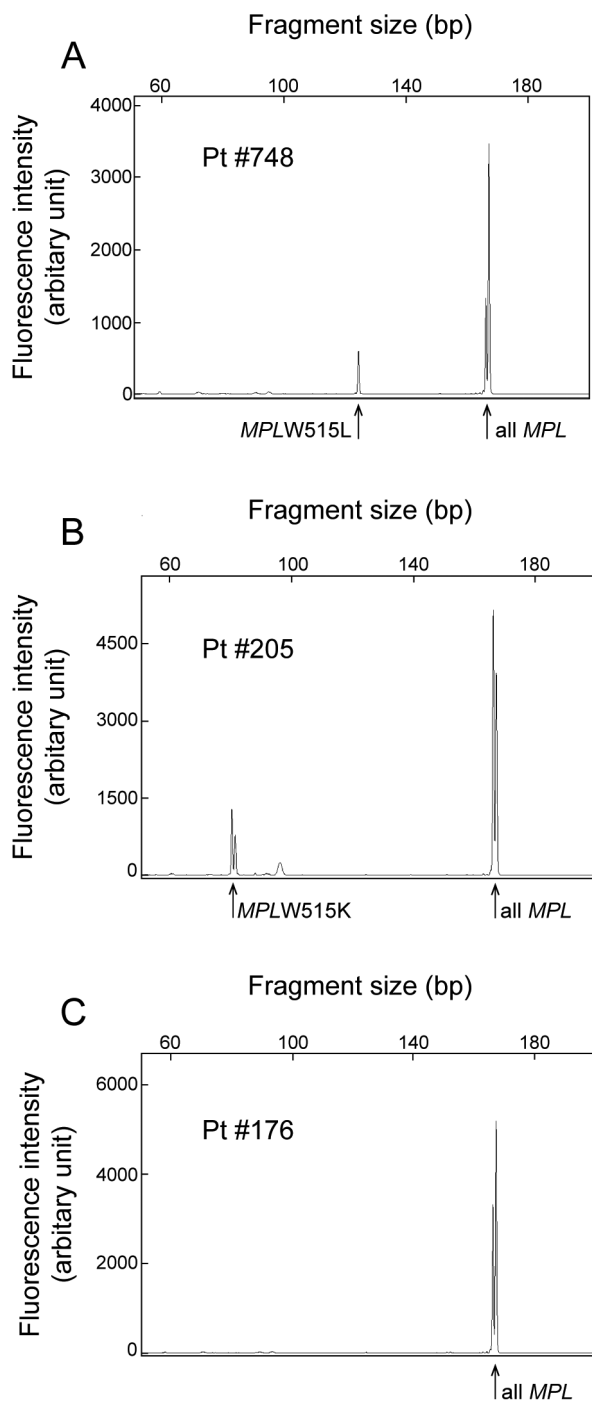
#### Human specimens

Genomic DNA was purified from peripheral blood collected from 20 patients previously diagnosed with MPN at Juntendo University Hospital (Hongo, Tokyo, Japan) or other participating institutions [22]. This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee

of Juntendo University School of Medicine (IRB#21076). Written informed consent for the use of samples and clinical records was obtained from all the patients prior to sample collection. Genomic DNA was purified from 200  $\mu$ L of each blood sample using the QIAamp DNA Mini Kit (Qiagen). The genomic DNA concentrations were measured with the NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, USA), and the DNA was stored at  $-80^{\circ}\text{C}$  until use.

#### Targeted deep sequencing

A 169-bp fragment containing the human *MPL* sequence (43349278 to 43349446 of NC\_000001.11) was PCR-amplified from genomic or standard plasmid DNA using forward (5'-



**Figure 4. Detection of *MPL* mutations in human specimens.** Representative capillary electropherograms from the DARMS-PCR assay with human specimens are presented. Patients #748 (A), #205 (B), and #176 (C) harbored *MPLW515L*, *W515K*, and wild-type alleles, respectively. The calculated allele frequencies are presented in Table 1. doi:10.1371/journal.pone.0104958.g004

AGTAGGGGCTGGCTGGAT-3') and reverse (5'-GGTCACA-GAGCGAACCAAGA-3') primers (see above). The PCR products were subjected to agarose gel electrophoresis and were purified with the QIAquick gel extraction kit (Qiagen). Sample libraries were prepared with the TruSeq DNA LT Sample Prep Kit

(Illumina, San Diego, USA) according to the manufacturer's instructions, and the libraries were deep sequenced with a MiSeq benchtop sequencer (Illumina). The data analysis was performed using CLC Genomics Workbench software version 6.5 (CLC Bio, Aarhus, Denmark) with a minimum coverage of 150,000 and a minimum variant frequency of 1%. The mutant allele frequency was calculated by dividing the number of mutant sequence reads by the sum of the mutant and wild-type reads.

## Results and Discussion

### Establishment of DARMS-PCR

To develop a single PCR assay for detecting the two major mutant alleles, *MPLW515L* (G1544T) and *W515K* (TG1543\_1544AA), we modified an ARMS-PCR method [6] by adding a total of four primers to each reaction; we renamed the method DARMS-PCR. Two mutant-specific primers (F\_inner and R\_inner) and two common primers (F\_outer and R\_outer) were designed (Figure 1A and B). *MPLG1544T*, TG1543\_1544AA, and a control fragment for all *MPL* alleles generated unique PCR products that were 128, 84, and 169 bp in size, respectively (Figure 1B). The two reverse primers (R\_inner and R\_outer) were labeled with the fluorescent dye FAM (5-carboxyfluorescein hydrate), which allowed us to determine the lengths and quantity of the PCR products using a capillary sequencer. According to the design of the assay, when a template containing a mixture of purified DNA fragments representing *MPLG1544T*, TG1543\_1544AA, and wild-type alleles was amplified under optimized conditions (see Materials and Methods), three PCR products corresponding to the different alleles were detected (Figure 1C). All three PCR products produced dual peaks, presumably because of an indefinite adenine addition at the end of each amplicon by Taq polymerase (Figure 1C). The sum of the peak height values from each dual peak was used in the following analyses.

### Determination of *MPL* mutant allele frequency using DARMS-PCR

To examine whether the *MPL* mutant allele frequency could be quantitatively determined using DARMS-PCR, we created a series of standard DNA templates containing different amounts of wild-type and mutant alleles. Templates containing 10, 20, 40, 60, 80, 90, or 100% mutant (*W515L* or *W515K*) allele DNA were used for DARMS-PCR, and the fluorescent intensities of the products were measured with the capillary sequencer (see Materials and Methods). As shown in Figure 2A and B, the fluorescence intensity of the mutant alleles over the intensity of all *MPL* alleles demonstrated a nearly linear correlation, indicating the accuracy of DARMS-PCR for quantitatively determining *MPL* mutant allele frequencies. Based on this result, we generated the following formulas to calculate the *MPL* mutant allele frequencies: *MPLW515L* allele frequency (%) =  $270 \times [\text{fluorescence value (arbitrary unit) for } MPLW515L \text{ PCR product}] / [\text{fluorescence value (arbitrary unit) for all } MPL \text{ allele PCR products}]$ ; and *MPLW515K* allele frequency (%) =  $200 \times [\text{fluorescence value (arbitrary unit) for } MPLW515K \text{ PCR product}] / [\text{fluorescence value (arbitrary unit) for all } MPL \text{ allele PCR products}]$ .

### Detection limit of *MPL* mutations using DARMS-PCR

We then examined the lower detection limit of DARMS-PCR under the conditions used for determining the mutant allele

**Table 1.** *MPL* allele frequencies determined using DARMS-PCR and deep sequencing.

<i>MPL</i> allele	Patientno.	<i>MPLW515L</i> allele frequency (%)*		<i>MPLW515K</i> allele frequency (%)*	
		DARMS-PCR	Deep sequencing	DARMS-PCR	Deep sequencing
<i>MPLW515L</i>	146	77.8	79.0	N.D.	0.0
	177	84.1	88.4	1.8	0.0
	526	24.7	17.1	N.D.	0.2
	748	33.4	43.7	N.D.	0.0
	782	19.4	47.4	0.9	0.0
	830	9.7	15.4	0.4	0.0
<i>MPLW515L/K</i>	127	N.D.	2.5	36.3	50.7
<i>MPLW515K</i>	120	N.D.	0.1	N.D.	1.0
	193	N.D.	0.1	22.2	33.6
	205	N.D.	0.1	45.4	35.0
Wild-type <i>MPL</i>	6	N.D.	0.0	N.D.	0.0
	50	N.D.	0.0	N.D.	0.1
	158	1.8	0.2	N.D.	0.0
	160	N.D.	0.0	0.5	0.0
	176	N.D.	0.6	N.D.	0.0
	185	N.D.	0.3	0.8	0.0
	199	N.D.	0.0	0.6	0.5
	607	0.7	0.0	N.D.	0.0
	688	1.0	0.1	N.D.	0.0
	710	N.D.	0.2	0.4	0.0

\*Allele frequencies below 1%, as determined using deep sequencing, were defined as negative for the assessed mutation. Note that allele frequencies below 5%, as determined using DARMS-PCR, were also defined as negative based on the results in Figure 3. N.D.: the corresponding peak was not detected.  
doi:10.1371/journal.pone.0104958.t001

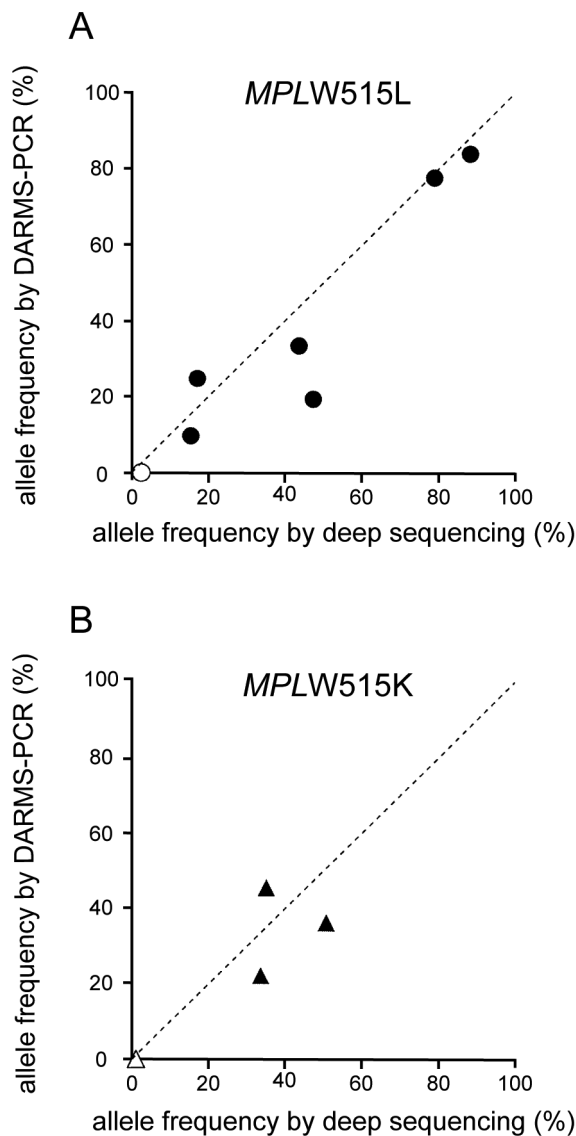
frequencies. We performed DARMS-PCR with DNA containing *MPL* mutant frequencies of 0, 1, 5, and 10%, and we obtained peak heights of 0, 55, 165, and 221 for *MPLW515L* and 0, 33, 199, and 731 for *W515K* (Figure 3). We then determined the average peak heights for nonspecific PCR products, which were defined as noise. The signal-to-noise (S/N) ratio was calculated as the fluorescence value for the peak of the mutant allele divided by the value for noise (Figure 3). When the allele frequency was 5%, the S/N ratios were 1.75 and 1.77 for *MPLW515L* and *W515K*, respectively. Thus, we concluded that the detection limit of DARMS-PCR was greater than 5% for both the *MPLW515L* and *W515K* mutations.

#### Validation of the DARMS-PCR assay results using deep sequencing

To examine the accuracy of DARMS-PCR for evaluating human specimens, we analyzed genomic DNA samples from 20 MPN patients whose *MPL* mutation status and allele frequencies had been determined using deep sequencing (see Materials and Methods). The formulas defined in Figure 2 were used to calculate the *MPL* mutant allele frequencies from the fluorescence peak values, which were determined using DARMS-PCR (Figure 4, Table 1). The results indicated that, using DARMS-PCR, 6 *MPLW515L*-positive and 3 *W515K*-positive MPN patients were successfully identified. Patient #120 was not identified due to having an *MPL* mutant frequency of 1.0% (according to deep

sequencing), which is below the DARMS-PCR detection limit (5%) (Table 1). Patient #127, who was initially identified as positive for *MPLW515K* using DARMS-PCR, was later found to be positive for both *MPLW515L* and *W515K* using deep sequencing (Table 1). The mutant allele frequency of *MPLW515L* in this patient was 2.5%, which was below the detection limit of DARMS-PCR. The remaining 10 patients, who were identified as wild-type for *MPL* using deep sequencing, had *MPL* allele frequencies below the positivity threshold of DARMS-PCR and thus were determined to be negative. Overall, although DARMS-PCR had a limitation of detection of mutant allele frequencies lower than 5%, all the patient specimens identified as having *MPL* mutations using DARMS-PCR were confirmed using deep sequencing. There was a strong correlation between the *MPL* mutant frequencies determined using DARMS-PCR versus deep sequencing, with errors ranging from -28.0% to 10.4% (Figure 5, Table 1). Further research is required to evaluate the quantitative nature of DARMS-PCR. Nevertheless, these data imply that DARMS-PCR can be used as a diagnostic tool to identify *MPL* mutations in MPN patients.

As observed with the standard template without mutant alleles (shown by the arrowhead at the *W515L* 0% panel in Figure 3) and with patient specimens that were defined as negative for *MPLW515K* or *W515L* with deep sequencing (Table 1), the DARMS-PCR generates false-positive PCR products and thus the subsequent appearance of low but clear allele frequencies. These



**Figure 5. Comparison of *MPL* mutant frequencies determined using DARMS-PCR and deep sequencing.** The horizontal axis represents the frequencies of the *MPLW515L* (A) or *W515K* (B) mutant alleles determined using deep sequencing, and the vertical axis represents the mutant allele frequencies determined using DARMS-PCR. The specific values are listed in Table 1. Filled symbols represent alleles identified using both DARMS-PCR and deep sequencing. Open symbols represent alleles identified only using deep sequencing. doi:10.1371/journal.pone.0104958.g005

false-positives presumably originate from faulty priming by inner primers that are capable of annealing to both mutant and

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wild-type alleles but are designed not to produce PCR products from the wild-type allele with introduced mutations (see Figure 1). Further optimization of the PCR program and primer sequences and positions is required to make the assay more sensitive and accurate. Although the deep-sequencer is apparently more accurate in terms of detecting the *MPLW515K/L* mutation, we observed potential error calls with less than 1% mutation frequency (see Table 1). This is likely the result of an erratic amplification during the sequencing sample preparation and/or a casual incident during the deep-sequencing. In addition, the current version of DARMS-PCR assesses only two major *MPL* mutations: *MPLW515K/L*, which account for approximately 75% of all *MPL* mutations [17]; thus, 25% of patients bearing rare *MPL* mutations, such as *MPLW515S* and *MPLS505N* [17], cannot be detected with this method. The incorporation of more primers into current DARMS-PCR and/or the establishment of more DARMS-PCR protocols for detecting other rare *MPL* mutations is required for more comprehensive assessment of *MPL* mutations.

In summary, we have developed and validated DARMS-PCR, a cost-effective, rapid, and accurate method for identifying two major *MPL* mutations with a single PCR assay. Although deep sequencing can be the most accurate method for detecting mutant alleles and measuring allele frequencies, it is considerably more expensive and time-consuming than DARMS-PCR. Compared with the specialized thermal cycler equipped with a fluorescence detection system that is needed to perform a melting curve analysis [12,13], DARMS-PCR requires only a general thermal cycler and a capillary sequencer (or even an agarose gel electrophoresis apparatus). DARMS-PCR is unique in its ability to simultaneously detect two mutations in one single-tube PCR assay; moreover, this method can be applied to detect other mutations as well. By employing this assay, the identification of approximately 75% of MPN patients with *MPL* mutations should be possible (see Introduction); thus, the use of DARMS-PCR will enhance screening for patients with *MPLW515L/K* mutations and will deepen our understanding of the pathogenesis of MPN.

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## Author Contributions

Conceived and designed the experiments: HT SM ST NK. Performed the experiments: HT SM YE Y. Sunami YH. Analyzed the data: HT SM MA NN. Contributed reagents/materials/analysis tools: Y. Sekiguchi AO. Contributed to the writing of the manuscript: HT SM MA ST NK.

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